REMARKS

Claims 19-22, 31-34, 43 and 44 are now pending in the present application, claims 23-30 and 35-42 having been canceled above. Claims 19, 21, 31 33 and 43 are newly amended to clarify their language. Support for these amendments can be found in the claims as originally presented, as well as throughout the specification, e.g., at page 3, lines 12-19; and page 5, line 28, to page 6, line 10. No new matter has been added.

Applicant requests entry of the above amendment and allowance of the claims in view of the remarks in this Response.

Interview Summary

Applicant thanks Examiners Bristol and Blanchard for the courtesy extended during the telephone interview of August 28, 2008 with the undersigned. During the interview, Applicant's undersigned representative discussed with the Examiners the amendments set forth above (a draft of the amendments[1] having been sent to Examiner Bristol prior to the interview) and the arguments described below. Examiner Bristol stated in the interview that the amendments were sufficient to overcome the rejection for lack of enablement. After a discussion of the obviousness rejection over McGuinness et al., Examiner Bristol indicated she was inclined to withdraw the obviousness rejection as well. Regarding the rejection for lack of written description, Examiner Bristol described some remaining concerns with respect to the importance of the linker sequence for proper folding of the diabody. The remarks below address all of the grounds for rejection set forth in the Final Office action dated April 15, 2008 (the "Office action").

Claim rejections under 35 U.S.C. § 112, first paragraph: lack of written description

The Office action rejected claims 19-44 for alleged lack of written description, stating: "The claims encompass a genus of linker molecules which are not supported by the original specification." (Office Action at page 5.) The Office action continues: "Accordingly, there is insufficient written description encompassing any one of the three linkers above because the relevant identifying characteristics of the genus such as structure or physical and/or chemical

[1] The amendments shown here are largely as shown in the draft. Differences include alterations to the language of part (e) of each of claims 19, 21, 31, and 33 that were not in the draft amendment, but are necessary for consistency with the other amendments to these claims. Also, certain text of claim 43 that the draft amendment showed as deleted is not deleted in the present amendment.

characteristics are not set forth in the specification as filed, commensurate in scope with the claimed invention." (Office Action at page 7.) Page 8 of the Office Action concludes: "In the absence of structural characteristics that are shared by members of the genus of a 'linker' of a), b) or c); one of skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus." In support of her position, the Examiner relies upon *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993); *Regents of the University of California v. Eli Lilly and Co.* 119 F. 3d 1559, 43 USPQ2d 1398 (Fed.Cir. 1997); and *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991).

Applicant respectfully traverses this rejection. Written description is a question of fact, judged from the perspective of one of ordinary skill in the art as of the relevant filing date. See *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). Compliance with § 112 requires sufficient information in the specification to show that the inventor possessed the invention at the time of that original disclosure. See *Vas-Cath*, 935 F.2d at 1563-64 ("[T]he applicant must . . . convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention."); *Union Oil Co. of Cal. v. Atl. Richfield Co.*, 208 F.3d 989, 997 (Fed. Cir. 2000) ("The written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [the inventor] invented what is claimed.'" (citation omitted)).

More recently, the Federal Circuit has held that "(1) examples are not necessary to support the adequacy of a written description [;] (2) the written description standard may be met [ ] even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falkner v. Inglis,* 448 F.3d 1357, 79 U.S.P.Q.2d 1001 (Fed. Cir. 2006). In particular, the Federal Circuit stated in *Falkner* that:

> A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention... *Falkner* at 448 F.3d 1366, quoting from

LizardTech, Inc. v. Earth Resource Mapping, PTY, Inc., 424 F.3d 1336, 1345 (Fed. Cir. 2005).

To aid this discussion, Applicant has attached hereto, as Exhibits A and B, flow diagrams respectively illustrating an embodiment of claim 19 and an embodiment of claim 21. The diagrams contain simple schematic representations of the nucleotide sequences recited in the respective claims. The representations of the sequences are not to scale and represent only one of the several possible arrangements of variable domains (shown in cross hatch and labeled as **L1** (for light chain variable domain 1), **H1** (for heavy chain variable domain 1), **L2** (for light chain variable domain 2) and **H2** (for heavy chain variable domain 2)). The open boxes between the variable domains represent nucleotide linkers. Exhibit A shows a restriction site within the linker between **L1** and **H1** in construct (a) and an identical restriction site at each of the two ends of the construct of (b). Exhibit B shows two different kinds of restriction sites within the linker in construct (a) and the same two kinds of restriction sites at either end of the construct of (b). In each Exhibit, (c) and (d) show the results of treatment with restriction enzyme(s), and (e) shows the finally assembled construct.

Contrary to the Examiner's position, one of skill in the art would understand, based on the teachings provided in the specification, that the Applicant was in possession of the genus of linkers recited in the claims. As the specification indicates, the use of linkers in the engineering of scFv and multi-specific antibodies was routine in the art at the time the application was filed. (See for example, the published application at paragraphs [0005] and [0006].) Moreover, the "relevant structural characteristics" of such linkers were well-known. The Völkel reference (Völkel, *Protein Engineering* 14(10): 815-823 (2001)) cited by the Examiner and discussed in detail below, as well as references cited within Völkel, make it clear that, while linker <u>length</u> may be important for making functional diabodies, the linker <u>sequence</u> can vary widely without affecting diabody activity. Völkel tested various middle linkers ranging from 7 to 19 amino acid residues in length, finding functional diabodies with middle linkers having 9, 13, 15, 17, or 18 residues, with the majority of functional diabodies having a 15-residue middle linker. Völkel also tested various "A" and "B" (i.e., left and right) linkers ranging from 0 to 6 residues in length, finding functional diabodies with left linkers from 1 to 7 residues (the 7 residue linker

apparently having resulted from mispriming during PCR) and right linkers ranging from 4 to 6 residues. (Völkel did not test longer left and right linkers, so does not speak to whether linkers longer than 6 or 7 residues would also work.) As the Examiner recognized, Völkel's experiments revealed "no obvious bias toward a specific linker sequence," thereby confirming the results of at least two other studies on linker sequence (Völkel, page 822, right-hand column). *It is clear that linker sequence is not a critical factor in obtaining functional diabodies, and also that many if not most sequences would be expected to work*, provided that the linker length is appropriate. One of skill in the art would understand that the middle linker should be relatively long, to permit flexibility. In contrast, the left and right linkers should be relatively short, to restrict flexibility and thereby keep the linked domains from interacting with each other. All of this is well known in the art.

The description of the linkers, both in terms of their length and sequence, in Applicant's specification is consistent with what was known in the art regarding linkers at the time the application was filed. The linker length spans a range, it is true, but that range is narrow and is within the range recognized in the art. Literal support for the nucleotide linker length recited in the claims ("30 to 150 base pairs") is found in the specification at paragraph [0058], which also provides a functional description of such a "long linker" as being "a linker of a size that enables the antibody heavy chain and light chain variable domains to be present as a scFv when the domains combined with the linker are expressed in a phage library." In other words, a "long linker" by definition must be long enough to possess the flexibility needed to allow the two variable domains of the single chain to interact and form a functional scFv. Flexibility is the only function required of the long linker, since it is the attraction between the two variable domains, and not the nature of the residues in the linker, that drives the proper folding of the polypeptide into a functional scFv. Examples of narrower ranges of linker length provided at paragraph [0058] are 36 to 90 bp (encoding 12 to 30 amino acids) and 45 to 60 bp (encoding 15 to 20 amino acids). Paragraph [0006] explains that the linker between the light and heavy domains of a typical scFv library (e.g., the claim 19(a) "first antibody library" and the claim 19(b) "second antibody library") would be "about 15 residues" in length, i.e., encoded by about 45 bp. Paragraph [0053] mentions "about 20 residues" (i.e., encoded by 60 bp) as another example of a useful linker length in the starting scFv libraries. Also see the example illustrated

in Fig. 1 of the specification, which depicts the linkers in the two starting scFv libraries as being 15-20 amino acid residues in length (i.e, 45-60 bp). A linker length of about 15 residues or more allows the scFv molecule to fold in half, permitting the light chain domain of the scFv to interact with the heavy chain domain to produce a functional scFv that can be screened for binding activity. Völkel showed that a linker as short as 9 residues (encoded by 27 bp) could perform this function in a diabody. One would certainly expect longer linkers, up to and even longer than the 50 residue length that corresponds to the upper limit in the present claims (i.e., 150 bp) also to possess the requisite flexibility to function as the sole linker in an scFv or as the middle linker in a diabody.

Further information about linker length is provided at paragraph [0007], which says that the linker that ends up in the middle of the ultimate diabody (see, e.g., the second of the three linkers in the diabody illustrated at the bottom of Exhibit A) will be 15 or more amino acid residues in length (i.e., encoded by 45 or more bp); this relatively long length is to permit the diabody to fold in half so the two domains on the left side of the diabody (i.e., domains L1 and L2 in the diabody at the bottom of Exhibit A) can interact with the two domains on the right side of the diabody (i.e., domains H2 and H1 in the diabody at the bottom of Exhibit A). This teaching about appropriate middle linker length is consistent with the teachings of Völkel et al. To ensure that the two domains on the left side of the diabody do not interact with each other instead of with the domains on the right side, the linker between the two domains on the left side (i.e., the "left linker" between domains L1 and L2 in the Exhibit A diabody) is made shorter than 15 amino acid residues. Paragraph [0006] mentions "about five residues" as being one example, and paragraph [0058] indicates a preference for a linker encoded by 6 to 27 bp (i.e., 2 to 9 amino acid residues in length), or other narrower subsets within that range. (This is consistent with Völkel's teaching that a left linker of 1 to 7 residues would work.) The same is true of the linker between the two domains on the right side of the diabody: here Völkel demonstrated that a right linker with 4 to 6 residues would function properly, but again did not rule out longer or shorter ones.

The Examiner appears to require that Applicant identify particular linker sequences that "upon translation into a peptide linker allow for the proper folding of the sc diabody or scFv for antigen binding." (Office Action at page 5.) This requirement is inconsistent with what was

known in the art about the ability of diabodies to tolerate wide variation in linker sequence. In keeping with the results of Völkel and the references cited therein, the specification indicates that the linker sequence may vary "as long as it does not interfere with the expression of the antibody variable domains that are connected at both of its ends" (Paragraph [0058]). A long peptide linker is essentially just a passive and flexible "tether" between two domains to keep them from floating away from each other and permit them to interact, while a short peptide linker is relatively inflexible in order to prevent such an interaction. Neither role is sequence-specific. Diabodies are driven to fold into a functional conformation by the attractions between the light and heavy variable domains, and not by the particular sequences of the linkers. Furthermore, the fact that the claims presently require that the first and second libraries encode single chain antibodies that bind to their respective cognate antigen means that the linkers used in the libraries must, by definition, function appropriately.

The method of claim 19 requires that the linker nucleotide sequence of the first antibody library (the library of (a)) comprise a recognition site for a restriction enzyme, while the method of claim 21 specifies that the library of (a) comprise two restriction enzyme sites in the linker. Examples of restriction enzyme recognition sites are provided in paragraph [0054] and illustrated in Figures 1 and 2. One of skill in the art could readily use the restriction sites provided in the specification or any other appropriate ones found in the prior art.

The case law cited in the Office Action cannot support the present rejection because the facts in those cases are markedly different from the present facts. Each of the three cases, *Fiers v. Revel*, *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, and *University of California v. Eli Lilly and Co.*, dealt with claims to a novel gene sequence that was obtained at a time when cloning and sequencing a full-length cDNA had not become routine in the art. *Fiers* involved a three-way interference relating to a cDNA encoding a human fibroblast interferon beta polypeptide; the prevailing party was the first party to set forth the correct DNA sequence. In *Lilly*, the Applicants were attempting to claim a genus of considerable size ("vertebrate" insulin cDNA) having disclosed only the rat insulin cDNA sequence. (Written description was not at issue in *Amgen*, and Applicant requests that, if the Examiner intends to maintain the written description rejection based on *Amgen*, she explain her rationale for doing so.) In contrast to the *Fiers* and *Lilly* situations, the art is aware of many examples of peptide linkers suitable for use in scFv and

diabodies, and also is aware that the function of peptide linkers is a mechanical one that is relatively sequence-independent (so that design of new, functional linkers would be a trivial matter). Applicant has described the nucleotide linkers of the claims in terms of their length and inclusion of restriction sites, and provided three examples (SEQ ID NOs:1, 3 and 5). One of skill in the art would have no reason to doubt that the Applicant was in possession of the invention as claimed.

In view of the description in the specification and the routine use of linkers in the art, the skilled artisan would understand the full scope of claims 19, 21, 31, 33 and 43 and their dependents. Applicant therefore requests reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, for failure to satisfy the written description requirement.

Claim rejections under 35 U.S.C. § 112, first paragraph: lack of enablement

The Examiner rejected claims 19-44 for alleged lack of enablement, stating that:

> ...the specification, while being enabling for producing single chain diabody libraries and constructs comprising the single chain diabody and a scFv antibody library created from a shortened linker where the VH and VL domains against any one antigen are from the same parent antibody and where the corresponding VH and VL domains are paired within the polypeptide construct, does not reasonably provide enablement for producing any of the foregoing libraries from unpaired single variable domains where the domains are from different antibodies against the same antigen or different antibodies against different antigens. (Office Action at page 8.)

Without conceding that claims as previously presented fail to satisfy this requirement, and solely for the purposes of furthering prosecution, claims 23-30 and 35-42 have been cancelled so that the rejection as applied to these claims is now moot, and independent claims 19, 21, 31, 33 and 43 have been amended to clarify their language. Specifically, amended claims 19 and 21, which are drawn to methods of making a single chain diabody library, both require that the first antibody library of (a) encodes single chain antibodies that bind to a first antigen. This ensures that the paired light and heavy chain variable domains of the library of (a) *must be capable of functioning together to bind that antigen*. It is well known in the art that a given heavy chain variable domain frequently can function to bind its cognate antigen whether it is paired with a matching wild type light chain variable domain from the same antibody, or with a mutated

version of that matching light chain variable domain, or even, in some cases, with a light chain variable domain from a different antibody. See, e.g., Clackson et al., Nature 352:624-628 (1991), attached hereto as Exhibit C. Clackson et al. explains on page 624 that a phage library expressing random combinations of heavy and light chains is selected for binding to a given antigen; this selection produces "promiscuous combinations (where the same V gene is found with several different partners)." Ensuring that the library of (a) encodes single chain antibodies that bind to a first antigen is therefore a simple matter of either originally constructing the library in a way that ensure the library includes members that bind to the antigen, or constructing it in a more randomly combinatorial way and then screening to select for a subset of single chain antibodies that bind to the antigen. The same is true for the second antibody library of each of claims 19(b) and 21(b), as well as the first and second nucleic acids specified in amended claims 31 and 33 and the antibody library of amended claim 43.

During the August 28, 2008, interview, Examiner Bristol indicated that the above claim amendments would overcome the rejection for lack of enablement. Applicants respectfully request that the rejection be withdrawn.

Claim rejections under 35 U.S.C. § 103: obviousness

The Examiner rejected claims 19-22, 31-34, 43 and 44 as unpatentably obvious in view of McGuinness et al. (*Nat. Biotech.* 14:1149-1154 (1996)) and Völkel (*Protein Engineering* 14(10):815-823 (2001)). According to the Office action at page 14, McGuinness discloses at page 1150 methods for constructing an antibody phage display library where the V regions from antibodies are constructed into two pools of scFv repertoires, and then recombined into a diabody format that has a linker between the two scFv halves. This is not a correct interpretation of McGuinness. As pointed out in the response filed January 7, 2008, and again noted by Applicant's representative during the August 28, 2008, interview, the diabodies produced by McGuinness were two-chain diabodies, not the single-chain diabodies of claims 19, 21, 31 and 33. This is apparent from the text of McGuinness at page 1150, col.2, first paragraph, which describes the diabody construct as having two separate VH/VL coding sequences separated by a stop codon ("Stop") and a ribosome binding site ("rbs"); in addition, each of the VH/VL coding sequences is shown as having its own leader peptide ("Leader 1" and "Leader 2"). (See also

Figure 1 on page 1150, which provides a diagram of the same construct.) These clues indicate that, though the two coding sequences are on a single construct, they are separately translated into two distinct polypeptide chains that are not joined by a peptide linker as maintained by the Office action. This fact is further evidenced by the statement on page 1152, col.2, 3rd full paragraph, of McGuinness that "each clone produces two polypeptide chains."

During the telephonic interview, Examiner Bristol acknowledged this clear distinction over McGuinness, and stated that she was therefore "inclined" to withdraw the obviousness rejection. Applicant asks that the rejection indeed be withdrawn. As previously pointed out in the response filed January 7, 2008, McGuinness's methods of making constructs do not bear any significant resemblance to applicant's methods. For example, McGuinness does not disclose use of a first antibody library in which the sequences encoding the heavy and light chain variable domains are linked by a nucleotide linker containing a restriction site, as required by step (a) of claim 19. It follows that this reference also does not disclose a step of cleaving at that restriction site, as required by step (c). Furthermore, McGuinness does not disclose inserting a sequence encoding a second pair of heavy and light chain variable domains into the open restriction site, and certainly does not disclose generating a construct encoding a single polypeptide chain comprising all four variable domains, all as required by step (e). In fact, it is difficult to derive from McGuinness any teaching of relevance to the presently claimed invention.

Unlike McGuinness, Völkel does disclose constructs encoding single-chain diabodies. However, any similarity to applicant's library methods pretty much ends there. Völkel started with a known single chain diabody construct containing four variable domains (pAB1 scDb CEAGal), not a pair of libraries, each member of which encodes two variable domains, as required by steps (a) and (b) of claim 19. Thus, none of the subsequent steps of the claims, all of which require manipulations of those libraries, was carried out by Völkel. As Völkel was interested in determining the effect of linker length on function of the diabody, Völkel used several restriction enzyme sites located in the pAB1 scDb CEAGal construct's three linkers as a means to generate a library of constructs that varied solely in the lengths of the three linkers (page 816, columns 1-2; fig.2). Völkel did not ligate a nucleic acid fragment encoding two variable domains into a cleaved restriction site between two variable domains, as required by step (e) of claim 19, and provided no reason to contemplate doing so. (In fact, introducing extra

variable domains into her construct in that manner would have ruined her experiment, the purpose of which was simply to study the effect of linker length.) Accordingly, Völkel does not disclose any of the steps of the present claims. Since Völkel was not concerned with combining two two-variable-domain libraries to prepare a four-variable-domain, single chain diabody library, and in fact started out with a construct that already contained all four variable domains in a single chain, Völkel provides no incentive to make the considerable modifications that would be required to result in a method such as applicant's. Völkel certainly does not supply what is absent from McGuinness, and vice versa.

The above discussion focused on claim 19, for simplicity. However, the same considerations apply to independent claims 21, 31, and 33 as well. All of these claims involve generation of nucleic acids that encode single chain diabodies (either as a library or as a construct), so the teachings of McGuinness are equally irrelevant to all of them. All start with a first library (or first nucleic acid) encoding two variable domains and a second library (or second nucleic acid) encoding two variable domains, and specify a method for manipulating the libraries/nucleic acids and then ligating the members of the two libraries (or the two nucleic acids) together in a very particular way. Völkel teaches nothing at all like the claimed methods, and no reason to seek to accomplish what applicant has accomplished. There is no apparent reason to combine the teachings of the two references, and even if one were to combine the two references, it would not result in a method that includes any of the steps of claims 19-22 or 31-34.

Claims 43 and 44, which were rejected *en bloc* along with claims 19-22 and 31-34 as obvious in view of the combination of McGuinness and Völkel, actually present different issues than do the other claims discussed above. Claim 43 is drawn to a method that begins with an antibody library in which each member of the library comprises a first and a second nucleotide sequence, each sequence encoding a variable domain, the two nucleotide sequences being connected to each other by a linker of 30 to 150 base pairs comprising two or more cleavage sites for a restriction enzyme. After treating the library with the restriction enzyme to cleave the two or more sites within the linker, the cleaved product is self-ligated to generate a second antibody library in which the sequences encoding the variable domains are joined by a nucleotide linker that is shorter than the linker in the original library. The claimed method provides a convenient

way to shorten the peptide linker that joins the two variable domains encoded by each member of the library. Such a method would be useful, for example, if it is desired to make the peptide linker too short to permit intra-chain interaction between the two variable domains, thereby facilitating inter-chain interactions and consequently dimerization to form two-chain diabodies. See, e.g., the specification at pages 2-3, carryover paragraph.

Neither McGuinness nor Volkel, nor the two in combination, discloses such a method. McGuinness describes preparation of libraries encoding scFv, each scFv containing a VH and a VL domain joined by a 15-amino-acid peptide linker (page 1150, col.1, first paragraph). Two of the libraries were specific for an antigen referred to as "phOx," and a third was specific for an antigen referred to as "Dig". McGuinness did not suggest any reason one would want to start with a long linker in these libraries and then shorten it, and certainly did not disclose the presently claimed method of including two or more cleavage sites in the sequence encoding the linker, then using a restriction enzyme to cleave those sites, followed by self-ligation of the cleaved product to close up the gap created by the restriction enzyme digestion. Where McGuinness disclosed use of a "zero linker", it was to join a VH from a phOx library with a VL from the Dig library, and also to join a VL from a phOx library with a VH from the Dig library. (See Figure 2 and also the text at page 1150, col.2, to page 1151, col.1.) Two different methods for generating the constructs with the zero linker between shuffled variable domains are illustrated in Figure 2 of McGuinness. Neither of these methods starts with a long linker and clips it out to result in the zero linker. Rather, each appears to utilize no linker at all, at any point. Thus, McGuinness discloses no reason to carry out the method of claim 43.

Völkel does not compensate for these deficiencies. Völkel studied how varying the length and sequence of each of the three linkers (labeled A, M (for "middle"), and B) in a four-variable-domain single chain diabody would affect the function of the single chain diabody. Völkel started with a single, defined sc diabody construct containing four defined variable domains, arranged in the order VH1-VL2—VH2-VL1 (see Fig.2). As detailed on page 816, col.1-2 (carryover paragraph), treatment with different restriction enzymes and PCR amplification with primers of various lengths and sequences yielded eight different fragments that were mixed and ligated together in either one or four ligation reactions, to yield a library of constructs all encoding *the original set of just four variable domains*, but with different middle

linkers (M). Then various positive clones isolated from that library were digested with two other restriction enzymes and amplified with various primers of different lengths and sequences in order to introduce diversity in the length and sequence of the other two linkers (A and B). The resulting library was screened for ability to bind to the sc diabody's two cognate antigens.

Plainly, Völkel did not disclose a method that is anything like the claim 43 method. There are at least four glaring differences.

First, Völkel started with a single, defined construct, not an antibody library as required by the claim.

Second, when Völkel cleaved the linker between the sequences encoding the VL2 and VH2 (i.e., the middle linker), she did not then self-anneal the cleaved product, as required by the claim, but rather carried out PCR amplification with several different primers and then annealed the resulting mixture of PCR-amplified products.

Third, even if one were to equate the defined construct of Völkel to the library of step (a) of the claim, the "linker M" library produced by annealing Völkel's PCR-amplified products did not meet the limitation "each member of the second library comprising the first and the second nucleotide sequences joined by a linker than is shorter than the linker in the library of (a)." As shown in Table III on page 818, Völkel's mixture produced a library of single chain diabodies varying by middle linker length; some of these were shorter than the original 16-amino acid (i.e., 48 bp) "wt" middle linker, but others were longer: specifically, 17 or 18 amino acids.

Fourth, each of the other two linkers in Völkel's construct, the so-called "A" and "B" linkers, connected a heavy chain variable domain and a light chain variable domain that were not directed against the "same antigen," as required by claim 43, rather, one variable domain was directed against carcinoembryonic antigen (CEA) and the other against β galactosidase (see Fig. 2). Only linker "M" linked two variable domains directed against the "same" antigen (β galactosidase), as required by the claim. Accordingly, Völkel's manipulations of the A and B linkers also do not meet the criteria of claim 43.

Thus, for several reasons, Völkel's methods are quite different from that claimed in claim 43. Neither Völkel nor McGuinness provides any motivation to modify the methods of either reference to come anywhere near that presently claimed.

The Examiner has not established that McGuinness and Volkel, either alone or in combination, teach or suggest *all the limitations of the subject matter now claimed.* Nor do these references provide any motivation to modify each others' teachings to arrive at the presently claimed inventions. They disclose entirely different experiments done for entirely different reasons. In view of the foregoing, the Office is respectfully asked to reconsider and withdraw this ground for rejection.

Applicant submits that all claims are now in condition for allowance, and such action is requested. Please apply any charges or credits to deposit account 06-1050 referencing Attorney Docket No. 14875-134US1.

Respectfully submitted,

Date: January 13, 2009

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EXHIBIT A

EXHIBIT A
(CLAIM 19)

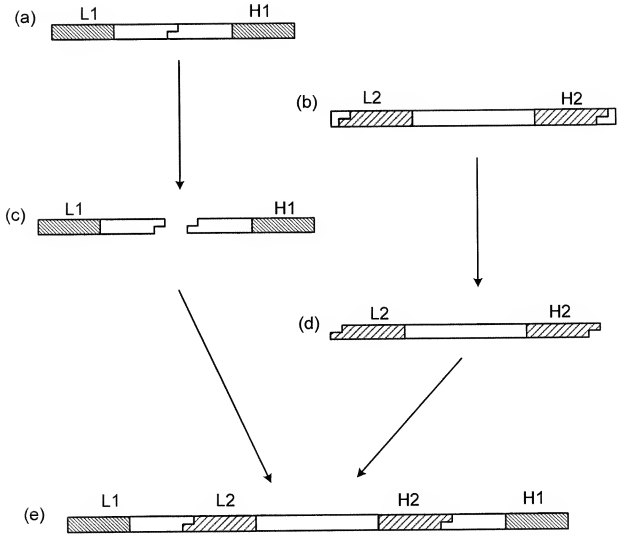

EXHIBIT B

EXHIBIT B
(CLAIM 21)

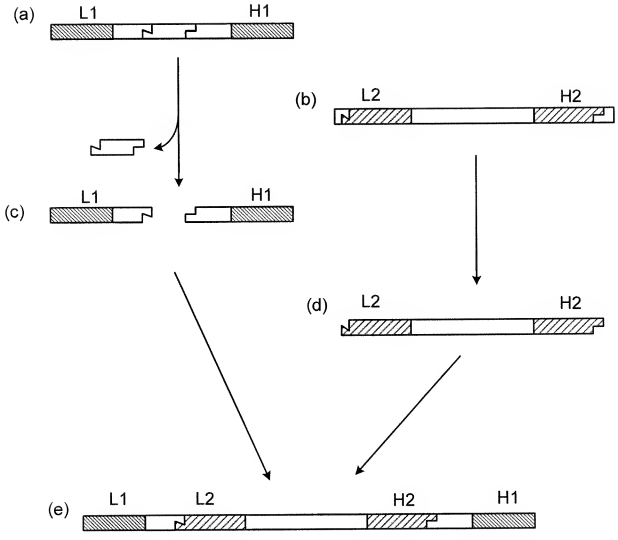

EXHIBIT C

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Making antibody fragments using phage display libraries

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To by-pass hybridoma technology and animal immunization, we are trying to build antibodies in bacteria by mimicking features of immune selection[1]. Recently we used fd phage[2] to display antibody fragments fused to a minor coat protein[3,4], allowing enrichment of phage with antigen[3]. Using a random combinatorial library of the rearranged heavy (V*H*) and kappa (V*κ*) light chains[5–8] from mice immune to the hapten 2-phenyloxazol-5-one (phOx), we have now displayed diverse libraries of antibody fragments on the surface of fd phage. After a single pass over a hapten affinity column, fd phage with a range of phOx binding activities were detected, at least one with high affinity (dissociation constant, $K_d = 10^{-8}$ M). A second pass enriched for the strong binders at the expense of the weak. The binders were encoded by *V* genes similar to those found in anti-phOx hybridomas but in promiscuous combinations (where the same *V* gene is found with several different partners). By combining a promiscuous V*H* or V*κ* gene with diverse repertoires of partners to create hierarchical libraries, we elicited many more pairings with strong binding activities. Phage display offers new ways of making antibodies from *V*-gene libraries, altering V-domain pairings and selecting for antibodies with good affinities.

We used the polymerase chain reaction (PCR)[9] to amplify the V*H* and V*κ* genes from the spleen messenger RNA of mice immunized with phOx, and also developed a 'PCR assembly' process[10] to link these genes together randomly for expression as single-chain Fv (scFv) fragments[11,12] (Fig. 1*a–c*). The assembled genes were cloned in a single step into the vector fdDOG1 (Fig. 1*e*) for display as a fusion with the fd gene III coat protein. This initial library of 2×10^7 clones seemed to be diverse (Fig. 1*d*), and sequencing revealed the presence of most V*H* groups[13] and V*κ* subgroups[14] (data not shown). None of the 568 clones tested bound to phOx as detected by enzyme-linked immunosorbent assay (ELISA).

The library of phages was passed down a phOx affinity column (Table 1*a*), and eluted with hapten. Of the eluted clones, 13%

TABLE 1 Affinity selection of hapten-binding phage

		Clones binding to phOx*		
	Precolumn	After first round	After second round	After third round
(*a*) Random combinatorial libraries				
phOx-immunized mice	0/568 (0%)	48/376 (13%)	175/188 (93%)	—
Unimmunized mice	—	—	0/388 (0%)	—
(*b*) Hierarchical libraries				
VH+B/V*κ*-*rep* library	6/190 (3%)	348/380 (92%)	—	—
VH-rep/V*κ*-d library	0/190 (0%)	23/380 (7%)	—	—
(*c*) Fractionation of VH-B/V*κ*-d and VH-B/V*κ*-b phage*				
Mixture of clones	88/1,896 (4.6%) (44/1,740 (2.5%)†)	55/95 (57.9%)	1,152/1,156 (99.7%)	1,296/1,299 (99.8%)

Selection of phage with hapten-binding activities from the random combinatorial and hierarchical libraries (*a* and *b*, respectively), and fractionation of clones with different affinities for phOx (*c*). For the random combinatorial libraries fdDOG1 RF was extensively digested with *Not*I and *Apa*LI, purified by electroelution[24] and 1 μg ligated to 0.5 μg (5 μg for the hierarchical libraries) of the assembled *scFv* genes in 1 ml with 8,000 units T4 DNA ligase (New England Biolabs) overnight at 16 °C. Purified ligation mix was electroporated in six aliquots into MC1061 cells[25] and plated on NZY medium[24] with 15 μg ml^{-1} tetracycline, in 243 × 243 mm dishes (Nunc); 90–95% of clones contained *scFv* genes by PCR screening (see legend to Fig. 1). Colonies were scraped into 50 ml 2 × TY medium[20] and shaken at 37 °C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10^{13} transducing units (TU) ml^{-1} in water (titred as in ref. 3). For affinity selection, a 1-ml column of phOx-BSA–Sepharose[27] M. Dreher and C. Milstein, unpublished results) was washed with 300 ml PBS, and 20 ml PBS containing 2% skimmed milk powder (MPBS). Phage (10^{12} TU) were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-ε-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP). About 10^6 TU eluted phage were amplified by infecting 1 ml log phase *E. coli* TG1 (ref. 28) and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 × TY medium and then processed as above. For the hierarchical libraries, V*H*-B and V*κ*-*d* genes were individually recloned, then assembled with the V*H* or V*κ* repertoires. For the fractionation of clone VH-B/V*κ*-d, 7×10^{12} TU phage in the ratio 20 VH-B/V*κ*-b:1 VH-B/V*κ*-d were loaded onto a phOx-BSA-Sepharose column in 10 ml MPBS and eluted as above. Eluted phage were used to reinfect *E. coli* TG1, and phage produced and harvested as before. About 10^{13} TU of phage were loaded onto a second affinity column and the process repeated to give a total of three column passes. Dilutions of eluted phage at each stage were plated in duplicate and probed separately[24] with oligonucleotides specific for V*κ*-b (5′-GAGCGGGTAACCACTGTACT) or V*κ*-d (5′-GAATGGTATAGTACTACCCT).

* In (*c*), numbers refer to VH-B/V*κ*-d.

† Numbers after three reinfections and cycles of growth. This control, omitting the column steps, confirms that a spurious growth or infectivity advantage was not responsible for the enrichment of clone VH-B/V*κ*-d.

bound to phOx, and ranged from poor to strong binding in ELISA. We sequenced 23 of these hapten-binding clones and found eight different V_H genes (A–H) in a variety of pairings with seven different V_K genes (a–g) (Fig. 2*a*). Most of the domains, such as VH-B and Vκ–d, were able to bind hapten with any of several partners[15]. The probability of finding multiple partners for a given chain should depend mainly on the inherent promiscuity of the chain and on the number of available partners and competing chains. Two other examples of promiscuous pairings have been noted in random combinatorial libraries made in λ phage[6,8], so this may prove to be a feature of small combinatorial libraries from immunized animals.

The sequences of the *V* genes were related to those seen in the secondary response to phOx, but with differences (Fig. 2*b*). Thus most phOx hybridomas from the secondary response use somatically mutated derivatives of three types of V_K genes, *V*κox1, '*V*κox-like' and *V*κ45.1 genes[16]. These can pair with V_H genes from several groups, but *V*κox1 more commonly pairs with the *V*Hox1 gene (V_H group 2; ref. 13). *V*κox1 genes are always, and *V*κox-like genes often, found in association with heavy chain genes (including *V*Hox1) that encode a short five-residue CDR3, with the sequence motif Asp-X-Gly-X-X (where X is any amino acid[16]), in which the central glycine creates a cavity for phOx (ref. 17). In our library, nearly all of the V_H genes belonged to group 1, and most of the V_K genes were ox-like and associated with V_H genes encoding a five-residue CDR3 motif Asp/Asn-X-Gly-X-X (Fig. 2*b*). *V*κox1 and *V*Hox1 were found only once (V_K–f and V_H-E), and not in combination with each other: indeed V_K-f does not encode the Trp 91 involved in phOx binding[17] and was paired with a V_H gene (V_H-C) encoding a six-residue CDR3.

The promiscuity of the VH-B and Vκ-d domains prompted us to force further pairings, by assembling these genes with the entire repertoires of either V_K or V_H genes from the same immunized mice. The resulting hierarchical libraries, (VH-B × *V*κ-*rep* and VH-*rep* × Vκ-d), each with 4×10^7 members, were subjected to a round of selection and hapten-binding clones isolated (Table 1*b*). Most were strong binders by ELISA

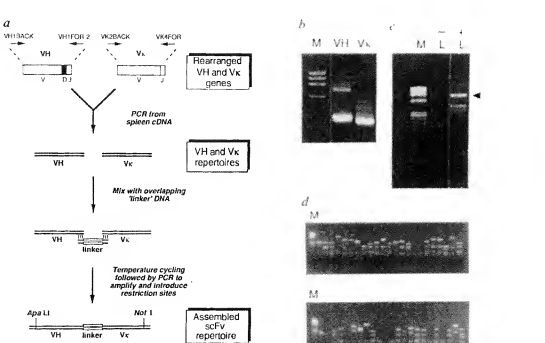

FIG. 1 PCR assembly of scFv library. *a*, V_H and V_K genes are separately amplified, then mixed with a linker fragment that overlaps them both. The linker (93 base pairs) encodes the short peptide $(Gly_4Ser)_3$, which links V_H and V_K in scFvs (ref. 11). Cycles of annealing–denaturation, followed by reamplification of the mixture, generate a random combinatorial cassette of V_H and V_K genes joined in-frame for expression. *b*, V_H and V_K gene repertoire PCR products from the immunized mice analysed by electrophoresis on agarose (1%) gel. *c*, PCR assembly of scFv gene repertoires with linker (+L) or without (−L); arrow indicates assembled repertoire. M is DNA marker ΦX174 replicative form DNA digested with *Hae*III. *d*, Diversity of library as seen by *Bst*NI fingerprinting of individual clones. *e*, Sequence of *fd* gene III around the signal peptide cleavage site in fdDOG1.

METHODS. For the random combinatorial libraries, cytoplasmic RNA was isolated[29] from the pooled spleens of either 5 male BALB/c mice boosted 8 weeks after primary immunization with phOx coupled to chicken serum albumin[27], or of 5 unimmunized mice. The cDNA was made with avian myoblastosis virus reverse transcriptase (Anglian Biotech)[30] and primers that straddle the junction between the variable and constant regions of γ heavy chains and κ light chains (C. Marks, unpublished data). V_H and V_K repertoires were amplified from the cDNA with 25 cycles of PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min) using Vent polymerase (New England Biolabs) and the primers VH1BACK (ref. 19) and VH1FOR-2 (ref. 31) or the primers VK2BACK and VK4FOR. The linker DNA was similarly amplified from pSW2scD1.3 (ref. 3) using primers LINKFOR and LINKBACK (complementary to VK2BACK and VH1FOR-2 respectively). After gel purification, 1 μg each of the V_H and V_K products were mixed with 300 ng linker in a 25 μl PCR reaction mix without primers, and cycled 7 times (94 °C 2 min, 72 °C 4 min) to join the fragments, then amplified for 20 cycles (94 °C 1.5 min, 72 °C 2.5 min) using 25 pmol each VH1BACK and VK4FOR primers. Finally, the assembled products were gel-purified and reamplified with VH1BACK-ApaLI and VK4FOR-NotI ('tagged' versions of the original primers) to append restriction sites. Products (1–5 μg) were extensively digested with *Apa*LI and *Not*I for cloning into fdDOG1. Recombinant colonies were screened by PCR[32] with the primers VH1BACK and VK4FOR, followed by digestion with the frequently cutting enzyme *Bst*NI. Primers VK2BACK 5′-GACATTGAGCTCACCCAGTCTCCA; VK4FOR, an equimolar mix of 5′-CCGTTTGATTTCCAGCTTGGTCCC, 5′-CCGTTTTATTTCCAGCTTGGTCCC, 5′-CCGTTTTATTTCAACTTTGTCCC and 5′-CCGTTTCAGCTCCAGCTTGGTCCC; LINKFOR, 5′-TGGAGACTGGGTGAGCTCAATGTC; LINKBACK, 5′-GGGACCACGGTCACCGTCTCCTCA; VH1BACK-ApaLI, as VH1BACK (ref. 19) but with 5′-CATGACCACAGTGCAC added at the 5′ end; VK4FOR-NotI, as VK4FOR but with 5′-GAGTCATTCTGCGGCCGC similarly added (restriction sites underlined).

FIG. 2 a Matrix of *V*H and *V*κ genes identified in phOx-binding clones selected from random combinatorial library. The number of clones found with each combination is shown. The binding to phOx–BSA, as judged by the ELISA signal, seemed to vary (marked by shading): no binding was seen to BSA alone. Optical density at 405 nm: 0.2–0.9, dotted box; 0.9–2.0, hatched box; >2.0, solid box. *b*, Encoded protein sequences of phOx-binding clones. Sequences of phOx-binding clones isolated (single-letter amino-acid code) after rounds of selection of the random combinatorial library, with pairings as above, or the hierarchical library. Note that the first eight or seven residues, and the last nine or eleven residues, of the *V*κ or *V*H genes, respectively, are encoded by the PCR primers. Classifications into VH groups[13] and *V*κ subgroups[14], and the position of residue 91 encoded by the *V*κ genes (*e*), are indicated. The relationship to genes from the hybridoma analysed secondary response to phOx (ref. 16) is also shown; all of the *V*κ genes are 'ox-like', apart from those marked * with an asterisk, which are *V*κox1, and the only example of VH group 2 (VH-E) is VHox1. The intensity of ELISA signals from the hierarchical libraries, corrected relative to the signal from control phage, are indicated: Optical density at 405 nm 0.9–2.0 (++), >2.0 (+++). Multiple isolations of sequences are marked, and sequences (*V*H-B and *V*κ-c) isolated from the random combinatorial library, and also the hierarchical libraries, are shown in italics. The VH-B/*V*κ-d and VH-B/*V*κ-c pairings gave similar signals (after correction of ELISA) when recovered from either combinatorial or hierarchical libraries.

METHODS. We screened for binding of the phage to haptens by ELISA: 96-well plates were coated with 10 μg ml^{-1} phOx–BSA[27] or 10 μg ml^{-1} BSA in PBS overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 μl 2×TY medium[28] with 12.5 μg ml^{-1} tetracycline in 96-well plates ('cell wells', Nuclon) and grown with shaking (300 r.p.m.) for 24 h at 37 °C. At this stage, cultures were saturated and phage titres were reproducible (10^{10} TU ml^{-1}). Phage supernatant (50 μl), mixed with 50 μl PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details given in ref. 3. To sequence the clones, template was prepared[24] from the supernatants of 10-ml cultures grown for 24 h, and sequenced using the dideoxy method[33] and a Sequenase kit (USB), with primer LINKFOR for the *V*H genes and primer fdSEQ1 (5′-GAATTTTCTGTATGA-GG) for the *V*κ genes.

a

Heavy chain

Light chain	A	B	C	D	E	F	G	H
a	2		1					
b		1		1	1			
c	1					1		
d		7	1		1			
e	2			2				
f			1					
g								1

b

VH sequences

From combinatorial library:

	CDR1	CDR2	CDR3	Group	ELISA signal
A	QVQLQQSGAELAKPGASVKMSCKASGYTFT SYTMH	WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN RYDAY	WGQGTTVTVSS x4	1	
B	QVQLQQSGAELAKPGASVKMSCKASGYTFT SYTMH	WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR WYGLY	WGQGTTVTVSS x9	1	
C	QVQLQQSGPELVRPGASVKISCKASGYTFT SYHWI	WVKQRPGQGLEWIG YINPYNDGTKYNEKFKG	KATLTSDKSSSTAYMELSSLTSEDSAVYYCAI YRSFFY	WGQGTTVTVSS x3	1	
D	QVQLQQSGPELVKPGASVKISCKASGYSFT GYTMN	WVKQSHGKSLEWIG RINPYNGGTFYNQKFKG	KATLTVDKSSSTAWMELLSLTSEDSAVYYCVG ITPFFAY	WGQGTTVTVSS x3	1	(see Fig.2a)
E	QVQLQQSGPELVKPGSSLSITCTVSGFSLS SYGVH	WVRQPPGKGLEWLG VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAN DRGDY	WGQGTTVTVSS	2	Fig.2a)
F	QVQLQQSGAELVRPGASVMMSCKASGYTFT SYNMH	WVKQTPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	
G	QVKLQQSGAELVRPGASVKLSCKASGYTFT SYNMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	EATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	
H	QVQLQQSGPELMKPGASVKISCKASGYSFS HNYMH	WVKQSHGKSLEWIG YIAPFNGGTTYNQKFKG	KATLTVDRSSSTAYMHLSSLTSEDSAVYYCAT DYGRD	WGQGTTVTVSS	1	

From hierarchical library *V*H-rep × *V*κ-d:

I	QVKLQQSGAELARPGVSMKISCKASGYTFT SYAMH	WVKQSQSKSLEWIG VISTYNGNTNYNQKFKG	KANTVTVDKSSSTAYMELARLTSEDSAIYYCAN DYGDY	WGQGTTVTVSS	1	+++
J	QVKLQQSGAELAKPGASVKMSCKASGYTFT SYTMH	WVKQRPGQGLEWIG YINPSSGYINYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR GRGAY	WGQGTTVTVSS	1	+++
K	QVQLQQSGAELAKPGASVKMSCKASGYTFT RDWMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN NYGLY	WGQGTTVTVSS x3	1	++~
L	QVQLQQSGLELAKPGASVKMSCKASGYTFT NYLMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN DYGYY	WGQGTTVTVSS x2	1	++~
M	QVQLQQSGAELARPGASVMKSCKASGYTFT RYWMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSDSAVYYCAR DYGYY	WGQGTTVTVSS	1	++~
N	QVQLQQSGAELVKPGASVKLSCKTSGYTFT SYIMH	WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN DYGYY	WGQGTTVTVSS	1	++
O	QVQLQQSGAELAKPGASVMMSCKASGYTFT SYIMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATFTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGAY	WGQGTTVTVSS	1	++
P	QVKLQQSGAELAKPGASVMKSCKASGYTFT SYWMH	WVKQRPGQGLELWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	++
Q	QVKLQQSGAELAKPGASVMKSCKATGYTFT SYLMH	WVKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	+++
R	QVQLQQSGAELAKPGASVMKSCKASGYTFT SYTMH	WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR WYGLY	WGQGTTVTVSS	1	++
S	QVQLQQSGAELARPGASVKMSCKASGYTFT TLMH	WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS x2	1	+++
T	QVQLQQSGAELARPGASVKMSCKASGYTFT SYTMH	WVKQRPGQGLEWIG YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS x6	1	++
U	QVKLQQSGAELAKPGASVKMSCKASGYTFT SYTMH	WVKQRPGQGLEWIG YINPCTGYTEYNQKFKD	KATLTADKSSTAYMQLSSLTSEDSAVYYCAR DYGYY	WGQGTTVTVSS	1	+++
R	*QVKLQQSGAELAKPGASVMKSCKASGYTFT RDWMH*	*WLKQRPGQGLEWIG YINPSTGYTEYNQKFKD*	*KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR WYGLY*	*WGQGTTVTVSS*	1	+++

*V*κ sequences

From combinatorial library:

	CDR1	CDR2	CDR3		
a	DIELTQSPSSLSASLGERVSLTC RASQEISGYLS	WLQQKPDGSIKRLLIY AASTLES	GVPKRFSGSKSGSDQYSLTISSLESEDFADYYC LQYASYPT	FGAGTKLELKRA x3	V	
b	DILTQSPAIMSASPGEKVTMTC RASSSVSSYLH	WYQQKSGASPKVWIT STSNLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQYSGYPLT	FGAGTKLELKRA x3	IV	
c	DILTQSPTTMAASPGEKITITC SASSISSSYLH	WYQQKPGFSPKLLIY RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC QQGSSIPLT	FGAGTKLELKRA x2	IV	(see
d	DILTQSPTTMAASPGEKITITC SASSISSSYLH	WFQQKPGFSPKLLIS RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC QQGSSIPLT	FGSGTKLELKRA x9	IV	Fig.2a)
e	DILTQSPAIMSASPGEKVTMTC SASSSVTYMH	WYQQKPGTSPKVWIY STSNLAS	GVPTRFSGSGSGTSYSLTISRMEAEDAATYYC QQRSSYPPT	FGSGTKLEIKRA x4	VI	
f	DILTQSPAIMSAVPGEKVTMTC SASSSVSYMH	WYQQKSGTSPKRWIY GTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC QQFSSNPLT	FGAGTKLELKRA	VI +	
g	DILTQSPAIMSASPGEKVTMTC SASSITMYNY	WYQQKPGASPKRWIY GTSKLAS	GVPARFSGSGSGTSTLTISSMEAEDAATYYC HQWSSYPWT	FGGGTKLEIKRA	VI	

From hierarchical library *V*H-B × *V*κ-rep:

h	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMN	WYQQKSGTSPKRWIY	GTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSSNPLT	FGAGTKLELKRA x4	VI +	+~+
i	DILTQSPAIMSASPGEKVTMTC	SASSSVSYIH	WFQQKPGTSPKLWIY	KTSNLAS	GVPARFSGSGSGTSYSLTISRMEAEDAATYYC	QQYHSYPLT	FGAGTKLELKRA	VI	~+~
j	DILTQSPTTMAASPGEKITITC	SASSISSSYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC	QQGSSIPLT	FGAGTKLELKRA	IV	+~+
k	DILTQSPTTMAASPGEMITITC	SATSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGAMEAEDVATYYC	QQGSSIPYT	FGAGTKLELKRA	IV	+~+
l	DILTQSPTTMAASPGEKITITC	SASSISSSYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDAATYYC	QQGSSIPLT	FGGGTKLEIKRA	IV	~+~
m	DILTQSPTTMAASPGEKITITC	SASSISSNHLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC	QQGSSIPLT	FGGGTKLEIKRA	IV	+~+
n	DILTQSPTTMAASPGEKITITC	SASSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC	QQGSSIPLT	FGGGTKLEIKRA	IV	++~
o	DILTQSPAIMAASPGEKITITC	SASSISSSYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC	QQGSSIPYT	FGGGTKLEIKRA	IV	+~+
p	DILTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	GTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSSNPLT	FGAGTKLELKRA x2	VI +	+++
q	DILTQSPAIMSASPGEKVTMTC	SASSSVTYMH	WYQQKSGTSPKRWIY	GTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSTNALT	FGAGTKLELKRA	VI +	++~
r	DILTQSPAIMSASPGEKVTMTC	RASSSVSSYLN	WYQQKSGASPKVWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLELKRA	IV	+++
s	DILTQSPAIMSASPGEKVTMTC	RASSSVSSYLH	WYQQKSGASPKVWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQRSSYPLT	FGAGTKLELKRA	IV	+++
u	DILTQSPAIMSASPGEKVTMTC	RASSSVSSYLH	WYQQKSGASPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLELKRA	IV	++
v	DILTQSPAIMSASPGEKVTMTC	RASSSVSSYLH	WYQQKSGASPKLWIY	STSNLPS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGGGTKLEIKRA	IV	++~
o	*DILTQSPTTMAASPGEKITITC*	*SASSISSSYLH*	*WYQQKPGFSPKLLIY*	*RTSNLAS*	*GVPARFSGSGSGTSYSLTITGTMEAEDVATYYC*	*QQGSSIPLT*	*FGAGTKLELKRA x3*	*IV*	*++*

(Fig. 2*b*). By sequencing 23 clones from each library, we identified 14 new partners for VH-B and 13 for Vκ-d; apart from VH-B and Vκ-c, none of the previous VH-B or Vκ-d partners (or indeed other partners) cloned and sequenced from the random combinatorial library was isolated again. These features are consistent with the much larger number of available partners (4×10^7) for the VH-B (or Vκ-d) domain, and the promiscuous nature of the domain. The *V*κ genes were mainly ox-like and the *V*H genes mainly group 1, but the only examples of *V*κ*ox*1 (Vκ-h, −p, −q and −r) encode Trp 91, and the VH-CDR3 motif Asp-X-Gly-X-X now predominates. Thus some features of the phOx hybridomas seem to emerge more strongly in the hierarchical library. The new partners differ from each other mainly by small alterations in the CDRs, indicating that much of the subtle diversity had not been tapped by the original random combinatorial library. More generally we find that a range of related antibodies can be made by keeping one of the partners fixed and varying the other, and this could be invaluable for fine tuning of antibody affinity and specificity.

To determine the range of antibody affinities for phOx, we recloned the combinations of VH-B with Vκ-b and Vκ-d (which gave weak and strong binding signals to phOx in ELISA) for secretion as soluble scFv fragments (Fig. 3, legend). Fluorescence quench titrations determined the K_d of VH-B/Vκ-d for phOx-GABA as 1.0×10^{-8} M (Fig. 3*a*), indicating that antibodies with affinities representative of the secondary response can be selected from phage display libraries. Indeed of anti-phOx hybridomas from the secondary response, only two (out of 11 characterized) secrete antibodies of a higher affinity than VH-B/Vκ-d (ref. 16). The K_d of VH-B/Vκ-b for phOx-GABA was determined as 1.8×10^{-9} M (Fig. 3*b*); thus phage bearing scFv fragments with weak affinities can also be selected with antigen, probably because of the avidity of the multiple antibody heads on the phage.

A second round of selection of the original, random combinatorial library from immune mice resulted in 93% of eluted clones binding phOx (Table 1*a*). Most of these clones were Vκ-d combinations, and bound strongly to phOx in ELISA (data not shown). Few weak binders were seen. This suggested that affinity chromatography had not only enriched for binders, but also for the best. To confirm this we mixed the phage VH-B/Vκ-d with a 20-fold excess of the phage VH-B/Vκ-b and subjected the mixture to rounds of selection: after only two rounds, essentially all the eluted phage were VH-B/Vκ-d (Table 1*c*).

We also constructed a random combinatorial library (2×10^6 members) from unimmunized mice, but found no phOx-binding clones after two rounds of selection (Table 1*a*). Immunization therefore seems to be necessary to create and/or enrich for VH or Vκ domains with at least some of the features required for hapten binding. With libraries of this size ($\sim10^6$ members), such domains need to be represented at a high frequency to reconstitute a binding site[1], and immunization ensures this by biasing the spleen lymphoid cell population heavily towards messenger RNA-rich blast cells making specific antibody (R. Hawkins and G.W., unpublished data). With larger libraries ($>10^7$) now accessible using selection[3] rather than screening[5–8], immunization may be unnecessary for the isolation of antibody fragments. It has been estimated that a library of 10^7 different antibodies will probably recognize >99% of epitopes with a dissociation constant of $\geq10^{-5}$ M (ref. 18), and we have shown here that we can recover antibody fragments with such affinities

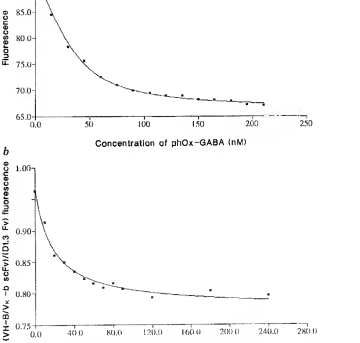

FIG. 3 Fluorescence quench titration of soluble scFv fragments. *a*, The K_d ($1.0\pm0.2\times10^{-8}$ M) for clone VH-B/Vκ-d was determined by fluorescence quench titration[34] of purified scFv (100 nM) with 4-γ-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA). Excitation was at 280 nm, emission was monitored at 340 nm and the K_d calculated as in refs 35 and 36. All values were calculated with standard errors included. The K_d was determined to be $1.0\pm0.2\times10^{-8}$ M with 0.38 ± 0.05 binding sites per scFv molecule. *b*, For measurement of the K_d of the low affinity clone VH-B/Vκ-b, 2 μM purified scFv protein was titrated with phOx-GABA as above. But to minimize light absorption by the higher concentrations of phOx-GABA required, excitation was at 260 nm and emission was monitored at 304 nm. In addition, the fluorescence values were divided by those from a parallel titration of the lysozyme binding Fv fragment D1.3 (ref. 31). The K_d was calculated as described in refs 34 and 36 and determined to be $1.8\pm0.3\times10^{-9}$ M, with a fractional quench of 0.20 ± 0.01.
METHODS. Clones VH-B/Vκ-b and VH-B/Vκ-d were reamplified with VK4FOR-*Not*I and VH1BACK-*Sfi*I (5′-CATGCCATGACTCGCGGCCCAGCCGGCCATGG-C(G/C)AGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG-3′), a primer that introduces an *Sfi*I site (underlined) at the 5′ end of the VH gene. *V*H-B/*V*κ-d was cloned into the phagemid pJM1 (A.D.G. and J. Marks, unpublished results) as an *Sfi*I–*Not*I cassette, downstream of the pelB leader for periplasmic secretion[37], with a C-terminal peptide tag for detection[31,38] and under the control of a λP_L promoter[39]. Cultures (10 l) of *Escherichia coli* N4830-1 (ref. 40) harbouring each phagemid were induced[26] and supernatants precipitated with 50% ammonium sulphate. The resuspended precipitate was dialysed into PBS, 0.2 mM EDTA (PBSE), loaded onto a 1.5-ml column of phOx-Sepharose[41] and the column washed sequentially with 100 ml PBS; 100 ml 0.1 M Tris–HCl, 0.5 M NaCl pH 8.0; 10 ml 50 mM citrate, pH 5.0; 10 ml 50 mM citrate, pH4.0 and 20 ml 50 mM glycine, pH 3.0. The scFv fragment was eluted with 50 mM glycine, pH 2.0, neutralized with Tris base and dialysed against PBSE. VH-B/Vκ-b was cloned into a phagemid vector (A.D.G., unpublished results) based on pUC119 (ref. 42) encoding identical signal and tag sequences to pJM1, and expression induced at 30 °C in a 10-culture of *E. coli* TG1 (ref. 28) harbouring the phagemid, as in ref. 43. The low affinity of clone VH-B/Vκ-b made its purification on phOx-Sepharose impossible. Therefore after concentration by ultrafiltration (Filtron, Flowgen) the supernatant (100 ml of 600 ml) was loaded onto a 1-ml column of protein A–Sepharose coupled[44] to the monoclonal antibody 9E10 that recognizes the C-terminal peptide tag[31,38]. The column was washed with 200 ml PBS and 50 ml PBS, 0.5 M NaCl. The scFv fragment was eluted with 100 ml 0.2M glycine, pH 3.0, with neutralization and dialysis as before.

from phage display libraries. The antibody fragments could be rebuilt from their genes into complete antibodies, and expressed in myeloma cells if required, as described in ref. 19.

It may be possible to retain the original VH/Vκ pairings of the splenocytes, as in hybridoma technology. In principle, PCR assembly could be used to construct such 'natural' libraries, if the V genes from individual cells could be amplified and assembled in capsules. More immediately, affinity selection from combinatorial and hierarchical libraries promises an attractive route to high-affinity antibodies, in particular those from humans that are difficult to produce by hybridoma technology[1]. But the use of phage display libraries is not limited to antibodies: it offers a powerful and general method to change and refine the properties of any other protein[20] or peptides[21–23] that can be displayed on the phage surface. □

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Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene

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A CYCLIC AMP-stimulated chloride conductance appears when the cystic fibrosis gene is expressed in non-epithelial cells by infection with recombinant viruses[1,2]. Cyclic AMP-stimulated conductance in this system is mediated by the same ohmic, low-conductance Cl^- channel as in human secretory epithelia[2–4], but control of this channel by phosphorylation has not been directly demonstrated. Here we report the appearance of the low-conductance Cl^- channel in Chinese hamster ovary cells after stable transfection with the cystic fibrosis gene. The channel is regulated on-cell by membrane-permeant analogues of cAMP and off-cell by protein kinases A and C and by alkaline phosphatase. These results are further evidence that the cystic fibrosis transmembrane regulator is a Cl^- channel which can be activated by specific phosphorylation events and inactivated by dephosphorylation; they reveal an unsuspected synergism between converging kinase regulatory pathways.

The coding sequence of the cystic fibrosis transmembrane regulator (CFTR) was cloned behind the metallothionein promoter of a plasmid that also contained a mutant dihydrofolate reductase gene, driven by the simian virus 40 early promoter (Fig. 1*a*). Stably transformed colonies were selected with methotrexate after calcium phosphate transfection of Chinese hamster ovary (CHO)-K1 cells. CFTR-expressing variants were chosen for further study on the basis of their capacity to produce a protein of the same apparent size as that present in T84 cell membranes in western blots probed with monoclonal antibodies against CFTR (Fig. 1*b*). CFTR protein was localized in a highly enriched plasma membrane vesicle fraction. In variants containing nearly the same amount of CFTR as T84 cells, cAMP-regulated chloride permeability, as monitored by ^{125}I efflux, was indistinguishable from that in T84 cells (Fig. 1*c*).

Patch-clamp recording was used to identify the channel responsible for cAMP-stimulated ^{125}I efflux. Channels became active in cell-attached patches after a lag of 69 ± 26 seconds when cells were exposed to membrane-permeant derivatives of cAMP; this was reversed by washing cAMP from the bath ($n = 7$, Fig. 2*a*). The channel was observed in 80% of all seals during cAMP stimulation (225/282) at an average density of between five and ten channels per patch. By contrast, it was recorded only once in 55 patches on unstimulated, CFTR-transfected cells and was never observed on cAMP-stimulated CHO cells that had been transfected with vector alone (0/31). Figure 2*b* shows that open probability was relatively independent of voltage, despite increased flickering at hyperpolarized potentials. Flickering was not observed using excised patches (see below), therefore these brief closures may reflect voltage-dependent, fast channel block by some anion in the cytosol. The current–voltage relationship rectified slightly in the outward direction during cell-attached recordings (Fig. 2*c*), but was linear ($r^2 = 0.9997$) when patches were excised and bathed symmetrically with 154 mM Cl^- (data not shown). In cell-attached patches the reversal potential (E_{rev}) was near the membrane potential (0.6 ± 0.3 mV applied potential) and the slope conductance at E_{rev} was 9.6 ± 0.5 pS ($n = 5$). The E_{rev} shifted to +32.4 ± 2.0 mV when the pipette solution contained 110 mM sodium gluconate and

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